



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Foxf2 is required for secondary palate development and Tgf signaling in palatal shelf mesenchyme

Citation for published version:

Nik, A, Johansson, J, Ghiami, M, Reyahi, A & Carlsson, P 2016, 'Foxf2 is required for secondary palate development and Tgf signaling in palatal shelf mesenchyme' *Developmental Biology*, vol. 415, no. 1, pp. 14-23. DOI: [doi:10.1016/j.ydbio.2016.05.013](https://doi.org/10.1016/j.ydbio.2016.05.013)

Digital Object Identifier (DOI):

[doi:10.1016/j.ydbio.2016.05.013](https://doi.org/10.1016/j.ydbio.2016.05.013)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Developmental Biology

Publisher Rights Statement:

Author's final peer-reviewed manuscript as accepted for publication.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



***Foxf2* is required for secondary palate development and Tgf β signaling in palatal shelf mesenchyme**

Ali M. Nik, Jeanette A. Johansson[#], Mozhgan Ghiami, Azadeh Reyahi, and Peter Carlsson*

1 Department of Chemistry and Molecular Biology, University of Gothenburg, Box 462, SE-405 30, Gothenburg, Sweden

Present address: University of Edinburgh Cancer Research UK Cancer Centre, MRC Institute for Genetics and Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XU, UK.

* Corresponding author: peter.carlsson@cmb.gu.se, +46 31 7863804

Running title: Foxf2 in palatal development

Abstract

The secondary palate separates the oral from the nasal cavity and its closure during embryonic development is sensitive to genetic perturbations. Mice with deleted *Foxf2*, encoding a forkhead transcription factor, are born with cleft palate, and an abnormal tongue morphology has been proposed as the underlying cause. Here, we show that *Foxf2*^{-/-} maxillary explants cultured *in vitro*, in the absence of tongue and mandible, failed to close the secondary palate. Proliferation and collagen content were decreased in *Foxf2*^{-/-} palatal shelf mesenchyme. Phosphorylation of Smad2/3 was reduced in mutant palatal shelf, diagnostic of attenuated canonical Tgfβ signaling, whereas phosphorylation of p38 was increased. The amount of Tgfβ2 protein was diminished, whereas the *Tgfb2* mRNA level was unaltered. Expression of several genes encoding extracellular proteins important for Tgfβ signaling were reduced in *Foxf2*^{-/-} palatal shelves: a fibronectin splice-isoform essential for formation of extracellular Tgfβ latency complexes; Tgfb3 – or betaglycan – which acts as a co-receptor and an extracellular reservoir of Tgfβ; and integrins αV and β1, which are both Tgfβ targets and required for activation of latent Tgfβ. Decreased proliferation and reduced extracellular matrix content are consistent with diminished Tgfβ signaling. We therefore propose that gene expression changes in palatal shelf mesenchyme that lead to reduced Tgfβ signaling contribute to cleft palate in *Foxf2*^{-/-} mice.

Introduction

Cleft palate, with or without cleft lip, is a common congenital malformation in humans with one out of 500 to 2500 newborns affected, depending on population. The high incidence reflects the complexity of the morphogenetic process and its underlying genetics; around 500 syndromes have been described that involve cleft palate (Dixon et al., 2011). Secondary palate development requires growth, elevation, and fusion of the palatal shelves in order to delimit the nasal from the oral cavity, and defects in any of these processes result in persistence of a cleft along the midline (Bush and Jiang, 2012).

In mouse, formation of the secondary palate begins at embryonic day 11.5 (E11.5) with palatal shelves growing out of the maxillary processes as anteroposterior ridges. The shelves are composed of ectomesenchyme, derived from cranial neural crest (Ito et al., 2003), and are covered by an ectodermal epithelium. Initially, the palatal shelves grow vertically down the sides of the tongue, but around E14 elevate to a horizontal position above the tongue and continue to grow horizontally, until they appose and fuse along the midline. A transient epithelial seam is formed which is gradually replaced by continuous mesenchyme (Vaziri Sani et al., 2005). Rostrally, the secondary palate fuses with the primary palate and the nasal septum, thereby creating the separation between the oral cavity and the two nostrils. In rodents, which are obligatory nose breathers, cleft palate is fatal due to its interference with breathing and suckling, and affected pups die shortly after birth with air filled guts. For a review of secondary palate development and disorders, see *e.g.* Bush and Jiang (2012).

Extracellular matrix (ECM) proteins are important for survival and continued proliferation of the palatal mesenchyme, as well as for expansion of the palatal shelves. Matrix accumulation and cell proliferation have been estimated to account for approximately equal shares of the volume increase of the palatal shelf tissue (Ferguson, 1988). The dominating ECM molecules in the developing palate are glycosaminoglycans (GAG), collagens and fibronectin. GAG consist mainly of hyaluronan, heparan sulphate and chondroitin sulphate, which hold up to ten times their mass in water and cause expansion of the palatal tissue through swelling (Foreman et al., 1991). This mechanism is utilized during elevation of the shelves from their initial vertical to their final

horizontal position, by asymmetrical deposition of hyaluronan (Ferguson, 1988; Moxham, 2003). The most abundant collagens are types I, III and V, which are distributed throughout the palatal mesenchyme, and IV which is associated with blood vessels and the sub-epithelial basement membrane (Ferguson, 1988; Foreman et al., 1991; Silver et al., 1981). Genetic and *in vitro* data show the importance of collagens for several steps in palatal development, including elevation, growth and fusion (Aszodi et al., 1998; Ferguson, 1988). Several paracrine factors stimulate the accumulation of ECM, where Tgf β increases production of collagens, whereas Egf mainly induces synthesis of GAG (Ferguson, 1988; Foreman et al., 1991; Moxham, 2003). However, since the secretion of matrix degrading metalloproteinases is controlled partly by the same factors (Miettinen et al., 1999), the net effect on ECM accumulation is not always obvious.

The transforming growth factor β isoforms Tgf β 1 and -3 are expressed in the palatal epithelium, whereas Tgf β 2 is found exclusively in the mesenchyme (Fitzpatrick et al., 1990; Pelton et al., 1990). Their receptors are present, and important, in both tissues: neural crest-specific inactivation of the Tgf β type II receptor gene, *Tgfr2*, causes cleft palate associated with reduced proliferation in the shelf mesenchyme (Ito et al., 2003), whereas conditional targeting of the same receptor in the epithelium instead hindered fusion of the palatal shelves by preventing elimination of the medial epithelial seam (Xu et al., 2006).

Fibroblast growth factors (Fgf) 8, 9, 10 and 18 are all required for normal palate development (Abu-Issa et al., 2002; Alappat et al., 2005; Colvin et al., 2001; Frank et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002; Rice et al., 2004) and signals through the receptors Fgfr1 and -2 (Rice et al., 2004; Trokovic et al., 2003). *Fgf10* is expressed in the mesenchyme, from where it activates expression of *Sonic hedgehog* (*Shh*) in the epithelium, which in turn targets the sub-epithelial mesenchyme (Lan and Jiang, 2009; Rice et al., 2004). *Shh* from the pharyngeal endoderm and the oral ectoderm is essential for proliferation and survival of cranial neural crest mesenchyme and induces patterning of the craniofacial area by activation of genes encoding forkhead transcription factors (Jeong et al., 2004).

The forkhead genes *Foxf1* and *Foxf2*, encode closely related transcription factors and are expressed in the splanchnic and extraembryonic mesoderm, and their derivatives (Aitola et al., 2000; Hellqvist et al., 1996; Mahlapuu et al., 1998; Peterson et al., 1997; Pierrou et al., 1994). In terms of expression level, *Foxf1* dominates during early embryogenesis and in the foregut, whereas both genes are highly expressed in the gut mesenchyme during organogenesis (Ormestad et al., 2004). *Foxf1* null mutants die at mid gestation due to severe defects in the extraembryonic vasculature (Astorga and Carlsson, 2007; Mahlapuu et al., 2001b), and heterozygotes suffer from a haploinsufficiency affecting the lungs and foregut (Kalinichenko et al., 2001; Mahlapuu et al., 2001a). *Foxf2* null mutants have developmental defects of the gut and die at birth (Ormestad et al., 2006). In adults *Foxf2* limits the size of the intestinal crypt stem cell niche through inhibition of Wnt signaling (Nik et al., 2013), and maintains the blood-brain barrier through activation of vascular Tgfb β and Pdgfr β signaling in CNS pericytes (Reyahi et al., 2015). Both *FoxF* genes are expressed in cranial neural crest cells of the maxillary and mandibular components of the branchial arches (Jeong et al., 2004), *Foxf2* as early as onset of the delamination of neural crest from the neural tube (Ormestad et al., 2004), whereas *Foxf1* is activated when the cells reach the branchial arches. Both the maintenance of *Foxf2* expression and the activation of *Foxf1* in cranial neural crest cells require Shh from the pharyngeal endoderm, or the oral ectoderm (Jeong et al., 2004; Lan and Jiang, 2009). Activation or maintenance by hedgehog signaling is a prerequisite for *FoxF* expression also in gut, lung, lateral plate and yolk sac mesoderm (Astorga and Carlsson, 2007; Madison et al., 2009; Mahlapuu et al., 2001a; Ormestad et al., 2006, and unpublished data).

Foxf2^{-/-} mutants die at birth with cleft secondary palate (Wang et al., 2003). The proposed mechanism suggests the mutant palatal tissue retains the same proliferation rate as in wild-type, but an abnormal tongue morphology prevents elevation of the palatal shelves, thereby obstructing palatal fusion (Wang et al., 2003). Here, we provide evidence for a defect intrinsic to the palatal shelf mesenchyme with *Foxf2* required for normal Tgfb β signaling, proliferation, and collagen accumulation.

Materials and Methods

Mouse strains

The *Foxf2* knockout (MGI:2667134) from the Miura lab (Wang et al., 2003) and our conditional *Foxf2^{fl}* allele (Reyahi et al., 2015) have been described elsewhere. A novel constitutive null allele was generated from *Foxf2^{fl}* by crossing with *Myh11-Cre* (Xin et al., 2002), which acts as a germ line deleter when maternally inherited. *Myh11-Cre* (MGI:2653286), *Wnt1-Cre* (MGI:2386570) and the double fluorescent reporter strain *mTmG* (MGI:3716464) were obtained from Jackson Lab (Maine). All strains were maintained on C57Bl/6J background.

Whole mount *in situ* hybridization and *lacZ* staining

Embryos were processed for whole mount *in situ* hybridization as described previously (Blixt et al., 2000) and hybridized with digoxigenin labeled antisense RNA probes for *Foxf1* (Mahlapuu et al., 2001b) and *Foxf2* (Ormestad et al., 2004). Vibratome sectioning of whole mount hybridized embryos was performed according to Landgren and Carlsson (2004).

BrdU analysis, immunohistochemistry and histology

For proliferation assays, E13.5 pregnant females were injected intraperitoneally with BrdU (Sigma; 100 µg/g body weight) and embryos were dissected and fixed exactly one hour later. Anti-BrdU (Pharmingen) was visualized by horseradish peroxidase (HRP) secondary antibodies and diaminobenzidine (DAB). BrdU positive and negative nuclei were counted in palatal shelf epithelium and in mesenchyme of tongue and palatal shelves from sections counterstained with Richardson's Methylene Blue/Azur II at different levels along the anteroposterior axis. Three intra-litter pairs of wild-type and *Foxf2^{-/-}* embryos were analyzed and the proliferation index for each level was based on the average of 6 slides.

For immunohistochemistry and histology, embryo heads were fixed in 4% buffered paraformaldehyde, and serially sectioned (coronal section, paraffin). Sections were stained with hematoxylin and eosin (H&E), or with antibodies directed against Foxf1

(R&D Systems), Foxf2 (R&D Systems), or collagen type I (Biomex). Antigen retrieval was carried out by pressure boiling in Tris-EDTA buffer pH 8.0.

Explant cultures

Heads of E13.5 embryos, from which the mandible, tongue and most of the brain had been removed, were cultured for 24 hours in rotating vials at 37°C in 5% CO₂, 100% humidity. During culture, the explants were suspended in serum-free F12 medium (Invitrogen), supplemented with streptomycin/penicillin (50U/ml), 0.2 mg/ml ascorbic acid and 0.1% BSA. Alternatively, palatal shelves were dissected and placed on MF filters (Millipore), supported by stainless grids in an air/medium interface and cultured for 48 hours in the same medium and atmosphere as for the suspension culture.

Extracellular matrix quantification

Palatal shelves were dissected from E13.5 embryos. Samples were weighed (fresh) and processed for measuring total collagen content with a soluble collagen assay (Sircol, Biocolor) following the manufacturers instructions. Quantification was made spectrophotometrically and calibrated with a collagen standard curve. GAG content was measured in a similar manner with a separate kit (Blyscan, Biocolor).

Western blot

Whole tissue extracts were prepared from E13.5 palatal shelves and protein concentration determined by Bradford assay (Biorad). Samples were run on polyacrylamide gels, blotted onto membranes and probed with antibodies against fibronectin (Sigma), Tgfβ2 (Santa Cruz), Smad2/3 (Cell Signaling), phosphorylated Smad 2/3 (Santa Cruz), p38 (Cell Signaling), phosphorylated p38 (Cell Signaling), and β-actin (mouse monoclonal, Sigma).

mRNA quantification by qPCR

Quantification of mRNA by PCR was performed on an Applied Biosystems instrument as described previously (Landgren et al., 2008). Briefly, cDNA synthesis was carried out using the Fermentas Revert AidTM Premium First Strand cDNA synthesis kit and analyzed with Power CYBR Green qPCR master mix from Applied Biosystems. Primers (Tab S1) were designed in house, or taken from online resources. Each sample was

analyzed in triplicate and normalized against the 36B4 transcript (Akamine et al., 2007). The number of biological replicates varied, but was never less than five per genotype.

Statistical analysis

Two-tailed Student's t-test with variances assumed equal, was used to compare averages. Fractions (percentages) were *arcsine* transformed when required to obtain a normal distribution of data points.

Results

***Foxf1* and *-2* expression partially overlap in the developing palate**

Both *FoxF* genes are expressed in the mesenchyme of the oral cavity and the tongue (Aitola et al., 2000; Jeong et al., 2004; Mahlapuu et al., 1998; Wang et al., 2003) and, given their partially redundant function in other organs (Ormestad et al., 2006), we wanted to investigate to what extent expression of *Foxf1* and *-f2* overlap in the developing palate. Whole mount *in situ* hybridization showed that *Foxf2* mRNA dominates in the posterior palatal shelves during E12.5–14.5, whereas *Foxf1* expression is more lateral, does not extend as far posteriorly, and is most prominent around tooth buds (Fig 1A, B). Upon fusion of the central part of the palate, at E14.5, *Foxf2* expression adopts a striped pattern, reflecting the development of the palatal rugae, and from E15.5 expression of both *FoxF* genes decreases significantly, with low expression persisting in the rugae. In addition to the palatal shelves, *Foxf2* mRNA is abundant in the ventral part of the nasal septum and in the tongue (Fig 1B, C).

The pattern observed by *in situ* hybridization was confirmed and further refined by immunohistochemistry on sections with antibodies to Foxf1 and -f2 (Fig 1D–K). Of particular interest is the difference in protein distribution in the posterior part, where Foxf1 staining is prominent around the tooth buds, but weak in the palatal shelves, whereas Foxf2 is abundant in the shelf mesenchyme. This difference is evident at E12.5, but particularly striking at E13.5 (compare bottom rows of Fig 1D/E and F/G). In contrast, the distribution of two proteins is similar in the maxilla, mandible and tongue at the level of the anterior molar bud, particularly at E12.5, but also at E13.5 (compare middle rows of Fig 1D/E and F/G). The tissue involved in palatal closure thus varies

considerably along the anteroposterior axis with respect to degree of overlap between *Foxf1* and *-f2* expression, but displays significant differences at the critical E13.5 stage.

At no stage or location were any of the FoxF proteins detected in epithelial cells, consistent with previous observations and the situation in other organs. The subepithelial mesenchyme of the tongue stained intensely for both proteins at all stages (E12.5–14.5) and at all levels along the anteroposterior axis (Fig 1D–K).

Cleft palate in *Foxf2*^{-/-} mutants

During analysis of the gut defect of E18.5 *Foxf2*^{-/-} mutants, we confirmed the cleft in the secondary palate (Fig 2D, F, L), previously described for this mutant (Wang et al., 2003). Wang et al (2003), reported complete penetrance for this malformation, whereas we initially observed a normal, fused palate in approximately 5% of *Foxf2* null mutants on C57Bl/6J background. During continued breeding on the same strain, the penetrance dropped to 80%, and has remained stable at this level for the last years. Genotypes are produced in normal Mendelian ratios and homozygous knockout embryos with and without the cleft palate occur in the same litters. In spite of extensive breeding, no *Foxf2*^{-/-} animals survived to the age when pups are genotyped (2–3 weeks). Presumably the gut and/or cerebrovascular defects are fatal even in cases where the palatal are not; an interpretation supported by the perinatal death of *Foxf1/Foxf2* compound heterozygotes, which have similar intestinal malformations, but normal palate (Ormestad et al., 2006).

Most of the analysis described in this paper was performed on the original *Foxf2* null allele from Miura and co-workers (Wang et al., 2003). We obtained a second constitutive null allele by Cre mediated germ line-deletion of exon 1 from our conditional *Foxf2* allele (Reyahi et al., 2015). A limited number of litters have been analyzed, but so far all E18.5 embryos homozygous for this null allele had cleft palate and appeared indistinguishable from the Miura null mutants. This confirms that clefting is caused by inactivation of *Foxf2*, rather than a linked secondary mutation.

A developmental defect intrinsic to the *Foxf2*^{-/-} palatal tissue

To investigate if the cleft palate of *Foxf2* null mutants involves altered development of the palatal shelves, rather than just obstruction by the tongue, we attempted to separate

the role of *Foxf2* in the two tissues. First, we conditionally targeted *Foxf2* in neural crest cells, using *Wnt1-Cre* (Danielian et al., 1998), which deletes *Foxf2* in the palatal shelf mesenchyme, while leaving it intact in all epithelia and in the mesodermally derived muscle cells of the tongue. Mice homozygous for the floxed allele (*Foxf2^{fl/fl}*) were crossed with *Foxf2^{-/+}; Wnt1-Cre*. The specificity of the Cre driver and the ontogeny of the relevant cell populations were verified by examination of E13.5 and E18.5 embryos double transgenic for *Wnt1-Cre* and the dual fluorescent marker *mTmG* (Muzumdar et al., 2007). The mesenchyme of the palatal shelves (E13.5) and secondary palate (E18.5) was completely recombined (green fluorescence; Fig 2I, J) with only blood vessels (which do not express *Foxf2* (Reyahi et al., 2015)) remaining non-recombined (red fluorescence). The oral, nasal and tongue epithelia also remained non-recombined, as did the muscle cells of the tongue. At E18.5 the tongue is dominated by muscle, but also contains connective tissue of neural crest origin (green fluorescence in Fig 2J). At E13.5 both cell types are present in the tongue, but the relative contribution of neural crest cells is significantly higher (Fig 2I). *Foxf2^{fl/-}; Wnt1-Cre* mutants developed cleft palate (Fig 2H, N), indistinguishable from that of the null mutant (Fig 2F, L), whereas all other genotypes in these litters, including *Foxf2^{fl/+}; Wnt1-Cre* and *Cre* negative *Foxf2^{fl/-}*, had closed palates (Fig 2G, M and data not shown).

Although this result showed that inactivation of *Foxf2* in the tongue musculature was not required for clefting, the substantial contribution of neural crest derived mesenchyme to the E13.5 tongue meant that alterations in tongue morphology might still be involved. Furthermore, the asymmetry in tongue morphology seen in many *Foxf2^{-/-}* mutants from E14.5 onwards (Fig 1K; 2D, F) occurred also in *Foxf2^{fl/-}; Wnt1-Cre* mutants (Fig 2H).

As an independent approach, we *in vitro*-cultured explants consisting of E13.5 heads, from which the mandible, the tongue and the majority of the brain had been removed. Deformation of the tissue was prevented by maintaining the explants in suspension through constant rotation. After 24 hours in culture, the palatal shelves of wild-type explants had fused in the central portion of the palate, with anterior and posterior gaps remaining (Fig 2O). This corresponds to the stage of early E14.5 embryos and showed that palatal development *in vitro* under these culture conditions mimics that of the *in vivo* situation, but proceeds somewhat slower. In contrast to wild-type, *Foxf2^{-/-}* explants

remained open (Fig 2P). This result supports the notion of reduced growth in *Foxf2*^{-/-} palatal shelves, which causes failure to close even in the absence of the tongue. When E13.5 maxillary explants were instead cultured for twice the time (48 hours) on a permeable filter, which supports the tissue and facilitates horizontal growth, wild-type and *Foxf2*^{-/-} explants alike formed a continuous tissue of merged palatal shelves. Sections of these explants confirmed that fusion had occurred and that the midline epithelial seam had disappeared (Fig 2Q, R). The fate of the midline edge epithelium of *Foxf2* mutants is thus comparable to that of controls under conditions where growth of the palatal shelves is sufficient to close the gap. This is consistent with the apparently normal palate of the up to 20% of *Foxf2* null mutants in which clefting is not penetrant.

To understand through which mechanism *Foxf2* affects palate development, we next focused on the two processes known to be most important for palatal shelf expansion: cellular proliferation and accumulation of extracellular matrix.

Reduced collagen content in *Foxf2*^{-/-} palatal shelves

Accumulation of ECM is an important component of palatal growth and two classes of matrix molecules dominate quantitatively: collagens and glycosaminoglycans (GAG). Immunostaining of sections from E13.5 embryos with an antiserum against a fibrillar collagen (type I) showed this ECM molecule to be abundant and ubiquitously distributed in the palatal shelf mesenchyme (Fig 2S, left). The staining was somewhat weaker in the *Foxf2* mutant than in wild-type posterior palatal shelves (Fig 2S). To corroborate the immunostaining data with a quantitative method, we used a spectrophotometric assay to measure the total collagen content of E13.5 palatal shelf tissue. The wild-type palates contained significantly more (61%, $p=0.02$, $n=10$) collagen than the *Foxf2* mutant ($n=6$), per mg of tissue (Fig 2T). A similar assay was used to measure the content of GAG. The modest reduction in GAG content in the *Foxf2* mutant (20%) was not statistically significant ($p=0.22$; $n=5$ for each genotype). However, the inter-individual variation and limited sample size would allow a potentially relevant difference to go undetected.

Decreased mesenchymal proliferation in *Foxf2*^{-/-} palatal shelves

Up until approximately E13.5, development of the palatal shelves in *Foxf2*^{-/-} embryos paralleled that of their wild-type litter mates, but during the subsequent 24 hours there

was a clear difference in morphogenesis (Fig 2A–D). Hence, E13.5 offers the latest opportunity to analyze comparable tissues, but ought to reveal a difference in proliferation, if present. To quantify cell proliferation, E13.5 embryos were collected one hour after BrdU injection of pregnant females. Coronal sections at different levels along the anteroposterior axis were scored for BrdU-positive and negative nuclei in epithelium and mesenchyme of the palatal shelves, and in the tongue mesenchyme (Fig 3). BrdU index of tongue mesenchyme (from the dorsolateral corner of tongue cross sections at levels corresponding to A/D and B/E in Fig 3) did not differ between genotypes (Fig 3G). Proliferation in epithelium was identical between genotypes at the level of the first molar buds (middle level; B/E/H/J in Fig 3). At the posterior level (C/F/I/K in Fig 3) *Foxf2*^{-/-} epithelium had fewer BrdU+ cells (14.3% vs 18.4% in wild-type), but the low number of cells meant higher stochastic variation and the difference was not statistically significant. In palatal shelf mesenchyme, proliferative cells were significantly fewer in *Foxf2*^{-/-} mutants at the posterior level (27.6% vs 39.0% in wild-type; p=0.0015; Fig 3C, F, G, I, K), but did not differ between genotypes at the middle level (26.2% vs 27.3%; Fig 3B, E, G, H, J). Posterior shelf mesenchyme was scored separately adjacent to the oral and nasal epithelia, but did not differ significantly (39.5% vs 28.2% for oral mesenchyme and 38.5% vs 27.0% for nasal) and the data were pooled for the analysis in Fig 3G.

Attenuated Tgfβ signaling in *Foxf2*^{-/-} palatal shelves

Decreased mesenchymal cell proliferation, together with a reduction in collagen accumulation, are likely proximate causes of cleft palate in *Foxf2* mutants. The link to *Foxf2* could either be direct, cell autonomous, or be mediated by any of the paracrine signaling pathways that are important for proliferation and matrix production in the developing palate. Given the important role of the Tgfβ pathway for palatal mesenchyme proliferation and collagen production, we investigated canonical Tgfβ signaling, measured as Smad2/3 phosphorylation by western blot. P-Smad2/3 was consistently attenuated in E13.5 *Foxf2*^{-/-} palatal shelves, whereas the level of total Smad2/3 protein was comparable to wild-type (Fig 4A). In contrast, the activity of the Smad independent Tgfβ pathway mediated by Traf6/Trak1/p38, which antagonizes the canonical pathway in palatal development (Iwata et al., 2012), was increased in *Foxf2* mutant palatal shelves,

as judged by phosphorylation of p38 (Fig 4A). Since Tgf β is both a mesenchymal mitogen (Ito et al., 2003) and an activator of collagen production in the developing palate (Foreman et al., 1991), and since abrogation of mesenchymal Tgf β signaling causes cleft palate (Ito et al., 2003), the reduced Smad2/3 phosphorylation is very likely to be relevant for the observed malformation in the *Foxf2* mutant.

Reduced level of Tgf β 2 in *Foxf2*^{-/-} palatal shelves

Of the three genes encoding Tgf β ligands, *Tgfb2* and -3, are important for palatal closure (Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997). *Tgfb3* is expressed exclusively in the epithelium, and its inactivation prevents fusion of the epithelia at the midline, without affecting palatal shelf growth (Proetzel et al., 1995). In contrast, *Tgfb2* is expressed in the palatal shelf mesenchyme and is required for proliferation and expansion of the shelves; disruption of this gene leads to palatal clefting associated with mesenchymal hypoplasia (Sanford et al., 1997). These distinct roles for Tgf β 2 and Tgf β 3 in mesenchyme and epithelium, respectively, are mirrored by the consequences of inactivating the Tgf β type II receptor gene, *Tgfr2*, in the shelf mesenchyme (Ito et al., 2003), vs targeting of the same receptor in the epithelium (Xu et al., 2006). A reduction in Tgf β 2 concentration thus appeared to be a plausible candidate for the cause of diminished Smad2/3 phosphorylation, mesenchymal hypoplasia, and reduced collagen content of *Foxf2* mutant shelves.

Western blot of extracts from E13.5 palatal shelves indeed showed a significant reduction in the amount of Tgf β 2 protein in *Foxf2*^{-/-} embryos (Fig 4A). With *Tgfb2* and *Foxf2* expressed in the same cells, the most straightforward explanation for this result would be for *Foxf2* to directly control the transcription of *Tgfb2*. However, the whole mount *in situ* hybridization described earlier showed no signs of reduced *Tgfb2* expression in *Foxf2*^{-/-} embryos, and quantification by qPCR confirmed a near wild type level of *Tgfb2* mRNA (mean \pm SEM: 83% \pm 7% of wild-type; n=23 wild-type, 24 *Foxf2*^{-/-}; p=0.11; Fig 4B). Hence, a post-transcriptional mechanism, such as translation, secretion, retention, or degradation, seemed likely to affect the concentration of biologically active Tgf β 2 protein, in a *Foxf2* dependent manner.

Reduced expression of betaglycan, integrins, tenascin and fibronectin in *Foxf2*^{-/-} palatal mesenchyme

Matrix metalloproteases (MMPs) regulate Tgfb signaling by cleaving the extracellular latency complexes (Ge and Greenspan, 2006), and could potentially be responsible for an altered amount of Tgfb protein in the tissue, without any effect on mRNA. We measured the total MMP activity in E13.5 palatal shelves by zymography, but found no difference in protease activity between extracts from *Foxf2*^{-/-} and wild-type palatal shelves (Fig S2). Thus, altered matrix protease activity does not appear to be responsible for the observed reduction of Tgfb2 protein in the *Foxf2* mutant.

Next, we focused on mechanisms of retention of Tgfb in the ECM. Tgfb dimers are sequestered in a large latency complex, consisting of a latency-associated peptide (Lap) and a latent Tgfb binding protein (Ltbp), covalently linked to fibronectin and fibrillin (Ten Dijke and Arthur, 2007). Fibronectin is critically required for formation of the latency complex; fibronectin deficient cells fail to incorporate Ltbp in the ECM, or to activate the Tgfb pathway in response to factors that mobilize latent Tgfb (Dallas et al., 2005; Fontana et al., 2005). The *Fnl* gene, encoding fibronectin, contains several alternatively spliced exons, generating a total of over 20 distinct peptide sequences. One of these, the ED-A exon, encodes an integrin binding site and is always absent from plasma fibronectin synthesized by the liver, but may or may not be included in tissue versions of the protein (Liao et al., 2002). Plasma (*i.e.* ΔED-A) fibronectin could efficiently rescue Ltbp incorporation into the ECM of *Fnl* null fibroblasts, whereas a range of other fibronectin peptides, including a 160 kDa protein that spans all the type III repeats and the variable region, but includes the ED-A domain, failed to do so (Dallas et al., 2005). The formation of Tgfb latency complexes thus appears to depend on fibronectin versions lacking the ED-A domain (Fig 4C).

Western blot showed a modest, but consistent, decrease in total fibronectin in E13.5 palatal shelves from *Foxf2*^{-/-} embryos, compared to wild-type litter mates (Fig 4D). The size difference between versions with and without the ED-A domain is too small to be resolved by western blot, but qPCR with primers specific for the +ED-A and ΔED-A

transcripts indicated unaltered levels of +ED-A mRNA, but a 55% decrease in Δ ED-A (Fig 4E; $p=0.01$; $n=11$ wild-type and 12 *Foxf2*^{-/-}).

In addition to its role in formation of the latency complex, fibronectin is also important for activation of Tgf β , as a receptor for integrins. Many integrins, including all α V-containing heterodimers, are involved in activation of latent Tgf β 1 and Tgf β 3, but not Tgf β 2, which lacks the RGD motif in the Lap peptide (Munger and Sheppard, 2011). The importance of this step for Tgf β signaling is illustrated by the Tgf β 1/ β 3 knockout phenocopy of mice lacking the Lap RGD motif (Yang et al., 2007), or integrin α V (Bader et al., 1998). Genes encoding several integrin chains, including the α V fibronectin receptor, are also transcriptional targets for Tgf β signaling, which forms the basis for a cooperative feed-forward loop of Tgf β and integrin signaling (Margadant and Sonnenberg, 2010; Pechkovsky et al., 2008).

Foxf2^{-/-} palatal shelves had reduced amounts of mRNA for both chains of the dominating integrin, α V β 1, although the reduction in *Itga5* was not statistically significant (*Itgb1*: 72% of wild type; $p<0.01$; *Itga5*: 60% of wild type; $p=0.08$; $n=8$ per genotype; Fig 4B). mRNA for *Itgb6* and *Itgb8* did not differ significantly between genotypes (Fig S1). mRNA for *Tgfbr3* and *Tnc* were also significantly reduced in *Foxf2*^{-/-} palatal shelves (Fig 4B). *Tnc* encodes tenascin c and *Tgfbr3* Tgf β receptor III, also known as betaglycan, both large extracellular chondroitin sulfate/heparan sulfate proteoglycans. Tenascins synergize with Tgf β signaling in collagen I biosynthesis, and like integrins they are targets of Tgf β signaling and participate in activation of latent Tgf β . The diverse roles of Tgfbr3 in Tgf β signaling are not fully understood (Bilandzic and Stenvers, 2012), but it binds Tgf β and presents ligand to the type II receptor (Tgfbr2). Notably, the requirement for Tgfbr3 for efficient signaling is most pronounced for Tgf β 2 (Lopez-Casillas et al., 1993), and defects in *Tgfbr3*^{-/-} mice have been attributed to defective signaling by Tgf β 2 (Sarraj et al., 2013). No change was seen in expression of *Tgfbr2* or *Alk5* (Fig S1).

Discussion

Foxf2 is a downstream target of an epithelio-mesenchymal crosstalk in the developing palatal shelf that starts with *Fgf10* from the mesenchyme activating *Shh* expression in the epithelium. *Shh* activates *Foxf2* expression in the mesenchyme and our results now link this pathway to expression of several genes of importance for Tgf β signaling, with consequences for collagen accumulation, and mesenchymal proliferation (Fig 5). Abrogation of Tgf β signaling in palatal shelf mesenchyme by targeting *Tgfb2* in neural crest leads to upregulation of *Foxf2* expression, which suggests a negative feedback mechanism. Importantly, genetic inactivation of many components of this chain, upstream and downstream of *Foxf2*, causes cleft palate with mesenchymal hypoplasia.

Significant reduction of proliferation at E13.5 was detected only in posterior shelf mesenchyme. This coincides with the tissue where *Foxf2* expression is highest, and where there is least overlap with expression of *Foxf1*. The results are thus consistent with partial redundancy of *Foxf1* and *-f2* in the anterior half of the developing palate.

The two factors that have been shown to be responsible for rapid expansion of the shelves between E13.5 and 14.5 — cellular proliferation and ECM accumulation — are both significantly reduced in the *Foxf2* mutant, which provides a plausible proximate explanation for the developmental defect. Likewise, the reduction in Smad2/3 phosphorylation and in Tgf β 2 protein level, are entirely consistent with the observed phenotype: Tgf β signaling is known to be a mitogen for palatal shelf mesenchyme and to stimulate collagen production. Furthermore, elimination of mesenchymal Tgf β signaling by targeting the only Tgf β ligand expressed in the mesenchyme, Tgf β 2, or the Tgf β receptor specifically in the mesenchyme, results in a cleft palate phenotype with hypoplastic shelves, very similar to the *Foxf2* mutant.

With mRNA for Tgf β 2 unaltered, the mechanism behind the reduction in Tgf β 2 protein is, however, far from obvious. A wealth of regulated steps intercalate between the transcription of *Tgfb* genes and their biological action, including secretion; intra- and extracellular proteolytic processing of pro-peptides; and formation, activation and degradation of latency complexes. Furthermore, many of these processes are regulated in a tissue specific way, and only partially understood. As a transcription factor with

potentially hundreds of target genes, *Foxf2* may influence palatal development in general, as well as *Tgfb* signaling in particular, in several ways.

One indication that several mechanisms may act in parallel is the observation that *Tgfb2* null mice have a lower penetrance of cleft palate (23%, Sanford et al., 1997) than *Foxf2*^{-/-} (80–100%). Strain background can have dramatic effects on penetrance (Mahlpuu et al., 2001a), and conclusions based on such differences should therefore be made with caution, but the higher incidence in *Foxf2* mutants suggests that inactivation of *Foxf2* may have pleiotropic effects on palatal development, of which a reduction in *Tgfb2* is one component. The lower penetrance of cleft palate in *Tgfb2*^{-/-} mice, compared to mesenchyme-specific inactivation of the receptor, suggest that *Tgfb*1 and *Tgfb*3 produced by the epithelium also target the mesenchymal cells. Activation of *Tgfb*1 and *Tgfb*3 require α V integrins, and the reduction of α V β 1 in *Foxf2* null palates may therefore aggravate the phenotype.

The majority of E18.5 *Foxf2*^{-/-} pups have a wide open gap between the nasal and oral cavities, whereas the remainder are, at least superficially, indistinguishable from wild-type; no intermediates have been observed. This indicates that *Foxf2* activity is limiting during a short period at the initiation of palatal shelf expansion, and for individuals that, for stochastic reasons, manage to pass through this stage with an above-threshold *Tgfb* signaling level, the remainder of palatal development appears to be robust. *Tgfb* is itself a potent activator of integrin and fibronectin expression (Hocevar et al., 1999; Varga et al., 1987). The positive feedback loop this creates (Margadant and Sonnenberg, 2010) is a likely mechanistic basis for the all-or-none palatal phenotype of *Foxf2* mutants.

During revision of this paper, Xu et al (2016) published an analysis of the role of *Foxf2* in palatal development. Their conclusion that *Foxf2* mutants have reduced proliferation in the palatal shelf mesenchyme that contributes to the cleft palate is virtually identical to ours. The genetic approach also suffers from the same limitation as ours, *i.e.* inability to delete *Foxf2* in the palatal shelf without also targeting some of the tongue mesenchyme, and persistent tongue abnormalities. In the second part of the paper, Xu et al show through an elegant set of experiments how FoxF proteins maintain epithelial *Shh* expression by inhibition of mesenchymal *Fgf18*. We confirmed their

observed increase of *Fgf18* mRNA in *Foxf2*^{-/-} E13.5 palatal shelves (158% ±4% of wild-type; p<0.01; Fig S1), whereas *Fgf10* mRNA did not differ significantly between genotypes (wild-type 100% ±7%, n=6; *Foxf2*^{-/-} 80% ±2%, n=7; mean ±SEM; p=0.08; Fig S1).

Acknowledgements

We thank N. Miura for the *Foxf2* knockout strain. This work was supported by grants to PC from the Swedish Cancer Foundation and the Swedish Medical Research Council (VR-M).

References

- Abu-Issa, R., Smyth, G., Smoak, I., Yamamura, K., Meyers, E.N., 2002. Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development* 129, 4613-4625.
- Aitola, M., Carlsson, P., Mahlapuu, M., Enerbäck, S., Peltö-Huikko, M., 2000. Forkhead transcription factor FoxF2 is expressed in mesodermal tissues involved in epithelio-mesenchymal interactions. *Dev Dyn* 18, 136-149.
- Akamine, R., Yamamoto, T., Watanabe, M., Yamazaki, N., Kataoka, M., Ishikawa, M., Ooie, T., Baba, Y., Shinohara, Y., 2007. Usefulness of the 5' region of the cDNA encoding acidic ribosomal phosphoprotein P0 conserved among rats, mice, and humans as a standard probe for gene expression analysis in different tissues and animal species. *J Biochem Biophys Methods* 70, 481-486.
- Alappat, S.R., Zhang, Z., Suzuki, K., Zhang, X., Liu, H., Jiang, R., Yamada, G., Chen, Y., 2005. The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice. *Dev Biol* 277, 102-113.
- Astorga, J., Carlsson, P., 2007. Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. *Development* 134, 3753-3761.
- Aszodi, A., Pfeifer, A., Wendel, M., Hiripi, L., Fassler, R., 1998. Mouse models for extracellular matrix diseases. *J Mol Med* 76, 238-252.
- Bader, B.L., Rayburn, H., Crowley, D., Hynes, R.O., 1998. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell* 95, 507-519.
- Bilandzic, M., Stenvers, K.L., 2012. Reprint of: Betaglycan: a multifunctional accessory. *Molecular and cellular endocrinology* 359, 13-22.
- Blixt, Å., Mahlapuu, M., Aitola, M., Peltö-Huikko, M., Enerbäck, S., Carlsson, P., 2000. A forkhead gene, *FoxE3*, is essential for lens epithelial proliferation and closure of the lens vesicle. *Genes Dev* 14, 245-254.

Bush, J.O., Jiang, R., 2012. Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. *Development* 139, 231-243.

Colvin, J.S., White, A.C., Pratt, S.J., Ornitz, D.M., 2001. Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. *Development* 128, 2095-2106.

Dallas, S.L., Sivakumar, P., Jones, C.J., Chen, Q., Peters, D.M., Mosher, D.F., Humphries, M.J., Kielty, C.M., 2005. Fibronectin regulates latent transforming growth factor-beta (TGF beta) by controlling matrix assembly of latent TGF beta-binding protein-1. *J Biol Chem* 280, 18871-18880.

Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K., McMahon, A.P., 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 8, 1323-1326.

Dixon, M.J., Marazita, M.L., Beaty, T.H., Murray, J.C., 2011. Cleft lip and palate: understanding genetic and environmental influences. *Nature reviews. Genetics* 12, 167-178.

Ferguson, M.W.J., 1988. Palate development. *Development* 103 Suppl, 41-60.

Fitzpatrick, D.R., Denhez, F., Kondaiah, P., Akhurst, R.J., 1990. Differential expression of TGF beta isoforms in murine palatogenesis. *Development* 109, 585-595.

Fontana, L., Chen, Y., Prijatelj, P., Sakai, T., Fassler, R., Sakai, L.Y., Rifkin, D.B., 2005. Fibronectin is required for integrin alpha5beta1-mediated activation of latent TGF-beta complexes containing LTBP-1. *FASEB J* 19, 1798-1808.

Foreman, D.M., Sharpe, P.M., Ferguson, M.W.J., 1991. Comparative Biochemistry of Mouse and Chick Secondary-Palate Development In vivo and In vitro with Particular Emphasis on Extracellular-Matrix Molecules and the Effects of Growth-Factors on Their Synthesis. *Arch Oral Biol* 36, 457-471.

Frank, D.U., Fotheringham, L.K., Brewer, J.A., Muglia, L.J., Tristani-Firouzi, M., Capecchi, M.R., Moon, A.M., 2002. An Fgf8 mouse mutant phenocopies human 22q11 deletion syndrome. *Development* 129, 4591-4603.

- Ge, G., Greenspan, D.S., 2006. BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. *J Cell Biol* 175, 111-120.
- Hellqvist, M., Mahlapuu, M., Samuelsson, L., Enerbäck, S., Carlsson, P., 1996. Differential activation of lung-specific genes by two forkhead proteins, FREAC-1 and FREAC-2. *J Biol Chem* 271, 4482-4490.
- Hocevar, B.A., Brown, T.L., Howe, P.H., 1999. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *Embo J* 18, 1345-1356.
- Ito, Y., Yeo, J.Y., Chytil, A., Han, J., Bringas, P., Nakajima, A., Shuler, C.F., Moses, H.L., Chai, Y., 2003. Conditional inactivation of *Tgfb2* in cranial neural crest causes cleft palate and calvaria defects. *Development* 130, 5269-5280.
- Iwata, J., Hacia, J.G., Suzuki, A., Sanchez-Lara, P.A., Urata, M., Chai, Y., 2012. Modulation of noncanonical TGF-beta signaling prevents cleft palate in *Tgfb2* mutant mice. *J Clin Invest* 122, 873-885.
- Jeong, J.H., Mao, J.H., Tenzen, T., Kottmann, A.H., McMahon, A.P., 2004. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Gene Dev* 18, 937-951.
- Kaartinen, V., Voncken, J.W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., Groffen, J., 1995. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 11, 415-421.
- Kalinichenko, V.V., Lim, L., Stolz, D.B., Shin, B., Rausa, F.M., Clark, J., Whitsett, J.A., Watkins, S.C., Costa, R.H., 2001. Defects in pulmonary vasculature and perinatal lung hemorrhage in mice heterozygous null for the Forkhead Box f1 transcription factor. *Dev Biol* 235, 489-506.
- Lan, Y., Jiang, R., 2009. Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* 136, 1387-1396.
- Landgren, H., Blixt, A., Carlsson, P., 2008. Persistent FoxE3 expression blocks cytoskeletal remodeling and organelle degradation during lens fiber differentiation. *Investigative Ophthalmology & Visual Science* 49, 4269-4277.

- Landgren, H., Carlsson, P., 2004. FoxJ3, a novel mammalian forkhead gene expressed in neuroectoderm, neural crest, and myotome. *Dev Dynam* 231, 396-401.
- Liao, Y.-F., Gotwals, P.J., Koteliensky, V.E., Sheppard, D., Van De Water, L., 2002. The EIIIA segment of fibronectin is a ligand for integrins $\alpha 9\beta 1$ and $\alpha 4\beta 1$ providing a novel mechanism for regulating cell adhesion by alternative splicing. *J Biol Chem* 277, 14467-14474.
- Liu, Z., Xu, J., Colvin, J.S., Ornitz, D.M., 2002. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev* 16, 859-869.
- Lopez-Casillas, F., Wrana, J.L., Massague, J., 1993. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 73, 1435-1444.
- Madison, B.B., McKenna, L.B., Dolson, D., Epstein, D.J., Kaestner, K.H., 2009. FoxF1 and FoxL1 link hedgehog signaling and the control of epithelial proliferation in the developing stomach and intestine. *J Biol Chem* 284, 5936-5944.
- Mahlapuu, M., Enerbäck, S., Carlsson, P., 2001a. Haploinsufficiency of the forkhead gene *Foxf1*, a target for Sonic hedgehog signaling, causes lung and foregut malformations. *Development* 128, 2397-2406.
- Mahlapuu, M., Ormestad, M., Enerbäck, S., Carlsson, P., 2001b. The forkhead transcription factor *FoxF1* is required for differentiation of extraembryonic and lateral plate mesoderm. *Development* 128, 155-166.
- Mahlapuu, M., Peltö-Huikko, M., Aitola, M., Enerbäck, S., Carlsson, P., 1998. FREAC-1 contains a cell type-specific transcriptional activation domain and is expressed in epithelial-mesenchymal interfaces. *Dev Biol* 202, 183-195.
- Margadant, C., Sonnenberg, A., 2010. Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO reports* 11, 97-105.
- Miettinen, P.J., Chin, J.R., Shum, L., Slavkin, H.C., Shuler, C.F., Derynck, R., Werb, Z., 1999. Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat Genet* 22, 69-73.

Moxham, B.J., 2003. The development of the palate — a brief review. *European Journal of Anatomy* 7, 53-74.

Munger, J.S., Sheppard, D., 2011. Cross talk among TGF-beta signaling pathways, integrins, and the extracellular matrix. *Cold Spring Harbor perspectives in biology* 3, a005017.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., Luo, L., 2007. A global double-fluorescent Cre reporter mouse. *Genesis* 45, 593-605.

Nik, A.M., Reyahi, A., Ponten, F., Carlsson, P., 2013. Foxf2 in Intestinal Fibroblasts Reduces Numbers of Lgr5(+) Stem Cells and Adenoma Formation by Inhibiting Wnt Signaling. *Gastroenterology* 144, 1001-1011.

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N., Takada, S., 2002. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev* 16, 870-879.

Ormestad, M., Astorga, J., Carlsson, P., 2004. Differences in the embryonic expression patterns of mouse Foxf1 and -2 match their distinct mutant phenotypes. *Dev Dynam* 229, 328-333.

Ormestad, M., Astorga, J., Landgren, H., Wang, T., Johansson, B.R., Miura, N., Carlsson, P., 2006. Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. *Development* 133, 833-843.

Pechkovsky, D.V., Scaffidi, A.K., Hackett, T.L., Ballard, J., Shaheen, F., Thompson, P.J., Thannickal, V.J., Knight, D.A., 2008. Transforming growth factor beta1 induces alphavbeta3 integrin expression in human lung fibroblasts via a beta3 integrin-, c-Src-, and p38 MAPK-dependent pathway. *J Biol Chem* 283, 12898-12908.

Pelton, R.W., Hogan, B.L., Miller, D.A., Moses, H.L., 1990. Differential expression of genes encoding TGFs beta 1, beta 2, and beta 3 during murine palate formation. *Dev Biol* 141, 456-460.

Peterson, R.S., Lim, L., Ye, H., Zhou, H., Overdier, D.G., Costa, R.H., 1997. The winged helix transcriptional activator HFH-8 is expressed in the mesoderm of the primitive streak stage of mouse embryos and its cellular derivatives. *Mech Dev* 69, 53-69.

Pierrou, S., Hellqvist, M., Samuelsson, L., Enerbäck, S., Carlsson, P., 1994. Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. *Embo J* 13, 5002-5012.

Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., Ferguson, M.W., Doetschman, T., 1995. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 11, 409-414.

Reyahi, A., Nik, A.M., Ghiami, M., Gritli-Linde, A., Ponten, F., Johansson, B.R., Carlsson, P., 2015. Foxf2 Is Required for Brain Pericyte Differentiation and Development and Maintenance of the Blood-Brain Barrier. *Developmental cell* 34, 19-32.

Rice, R., Spencer-Dene, B., Connor, E.C., Gritli-Linde, A., McMahon, A.P., Dickson, C., Thesleff, I., Rice, D.P.C., 2004. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* 113, 1692-1700.

Sanford, L.P., Ormsby, I., Gittenberger-de Groot, A.C., Sariola, H., Friedman, R., Boivin, G.P., Cardell, E.L., Doetschman, T., 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 124, 2659-2670.

Sarraj, M.A., Escalona, R.M., Western, P., Findlay, J.K., Stenvers, K.L., 2013. Effects of TGFbeta2 on wild-type and Tgfr3 knockout mouse fetal testis. *Biology of reproduction* 88, 66.

Silver, M., Foidart, J., Pratt, R., 1981. Distribution of Fibronectin and Collagen during Mouse Limb and Palate Development. *Differentiation* 18, 141-149.

Ten Dijke, P., Arthur, H.M., 2007. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol* 8, 857-869.

Trokovic, N., Trokovic, R., Mai, P., Partanen, J., 2003. Fgfr1 regulates patterning of the pharyngeal region. *Genes Dev* 17, 141-153.

- Varga, J., Rosenbloom, J., Jimenez, S.A., 1987. Transforming growth factor beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. *Biochem J* 247, 597-604.
- Vaziri Sani, F., Hallberg, K., Harfe, B.D., McMahon, A.P., Linde, A., Gritli-Linde, A., 2005. Fate-mapping of the epithelial seam during palatal fusion rules out epithelial-mesenchymal transformation. *Dev Biol* 285, 490-495.
- Wang, T., Tamakoshi, T., Uezato, T., Shu, F., Kanzaki-Kato, N., Fu, Y., Koseki, H., Yoshida, N., Sugiyama, T., Miura, N., 2003. Forkhead transcription factor Foxf2 (LUN)-deficient mice exhibit abnormal development of secondary palate. *Dev Biol* 259, 83-94.
- Xin, H.B., Deng, K.Y., Rishniw, M., Ji, G., Kotlikoff, M.I., 2002. Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. *Physiol Genomics* 10, 211-215.
- Xu, J., Liu, H., Lan, Y., Aronow, B.J., Kalinichenko, V.V., Jiang, R., 2016. A Shh-Foxf-Fgf18-Shh Molecular Circuit Regulating Palate Development. *PLoS genetics* 12, e1005769.
- Xu, X., Han, J., Ito, Y., Bringas, P., Jr., Urata, M.M., Chai, Y., 2006. Cell autonomous requirement for Tgfb2 in the disappearance of medial edge epithelium during palatal fusion. *Dev Biol* 297, 238-248.
- Yang, Z., Mu, Z., Dabovic, B., Jurukovski, V., Yu, D., Sung, J., Xiong, X., Munger, J.S., 2007. Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. *J Cell Biol* 176, 787-793.

Figure legends

Figure 1

Expression of *Foxf1* and *Foxf2* around the oral cavity E12.5–15.5. A, B. Ventral views of maxilla, palatal shelves and palate of E12.5–15.5 embryos hybridized with *Foxf1* (A) and *Foxf2* (B) probes. C. Sections of E12.5 embryo hybridized with *Foxf2* probe. A–P = anteroposterior axis. D–K. Immunohistochemistry of E12.5 (D, E), E13.5 (F–H) and E14.5 (I–K) wild-type (D–G, I, J) and *Foxf2*^{-/-} (H, K) embryos with Foxf1 (D, F, H, I, K) and Foxf2 (E, G, J) antibodies at three levels along the A–P axis.

Figure 2

Cleft palate in *Foxf2*^{-/-} mutants. A–H. H&E stained sections of E13.5 (A, B), E14.5 (C, D), E18.5 (E, F) and P0 (G, H) wild-type (A, C, E), *Foxf2*^{-/-} (B, D, F), *Wnt1-Cre*; *Foxf2*^{fl/+} (G) and *Wnt1-Cre*; *Foxf2*^{fl/-} (H) heads at three levels along the A–P axis. I, J. Sections at three levels along the A–P axis of E13.5 (I) and E18.5 (J) heads of *Wnt1-Cre*; *mTmG* embryos (red = non-recombined, green = recombined). K–N. Ventral views of palates of E18.5 wild-type (K), *Foxf2*^{-/-} (L), *Wnt1-Cre*; *Foxf2*^{fl/+} (M) and *Wnt1-Cre*; *Foxf2*^{fl/-} (N) embryos. Arrowheads indicate closed (K, M) and open (L, N) secondary palates. O, P. Wild-type (O) and *Foxf2*^{-/-} (P) head explants without tongue and mandible cultured *in vitro* for 24 hours, starting at E13.5. Q, R. Sections of wild-type (Q) and *Foxf2*^{-/-} (R) palatal explants cultured *in vitro* on filters for 48 hours. Arrowheads indicate the midline. S. Immunohistochemical staining of collagen type I in E13.5 palatal shelves from wild-type (left) and *Foxf2*^{-/-} (right) embryos. T. Spectrophotometric quantification of collagen content in E13.5 palatal shelves. Means (61% more in wild-type) ±SEM, p=0.02, n=10 wild-type and 6 *Foxf2*^{-/-} embryos.

Figure 3

Analysis of proliferation in E13.5 embryos by BrdU incorporation. A–F. Sections of wild-type (A–C) and *Foxf2*^{-/-} (D–F) heads stained with anti-BrdU at three levels along the anteroposterior axis. G. Percentage BrdU+ cells in epithelium and mesenchyme of palatal shelves, and in tongue mesenchyme. Means of 3 intra-litter pairs of wild-type and

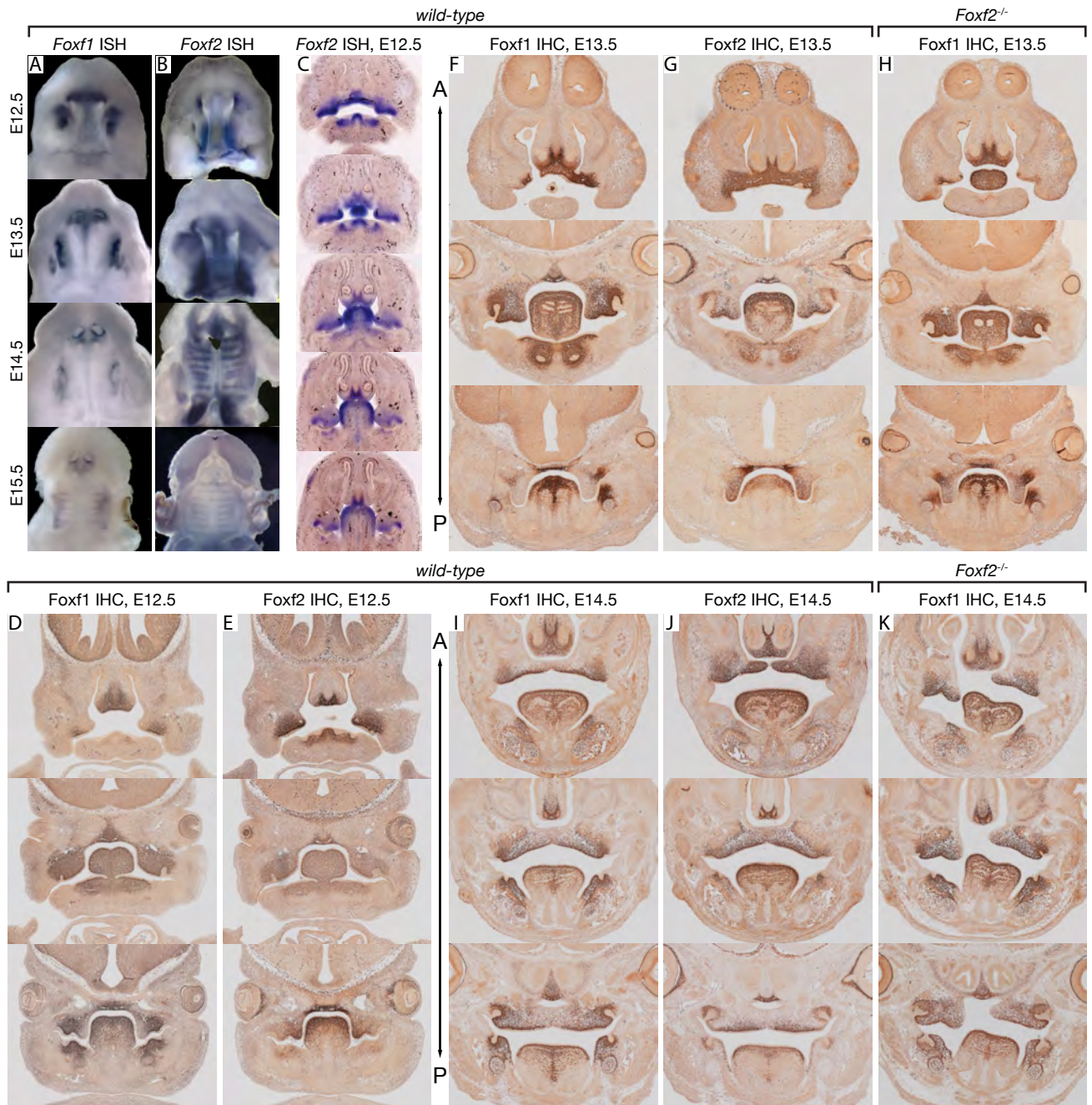
Foxf2^{-/-} embryos with value for each embryo and level based on 6 scored sections. Error bars = \pm SEM. Significant difference between genotypes only observed in posterior mesenchyme ($p=0.0015$). H–K. Close-ups of palatal shelves at the middle (first molar bud; H, J) and posterior (I, K) levels of wild-type (H, I) and *Foxf2*^{-/-} (J, K) embryos.

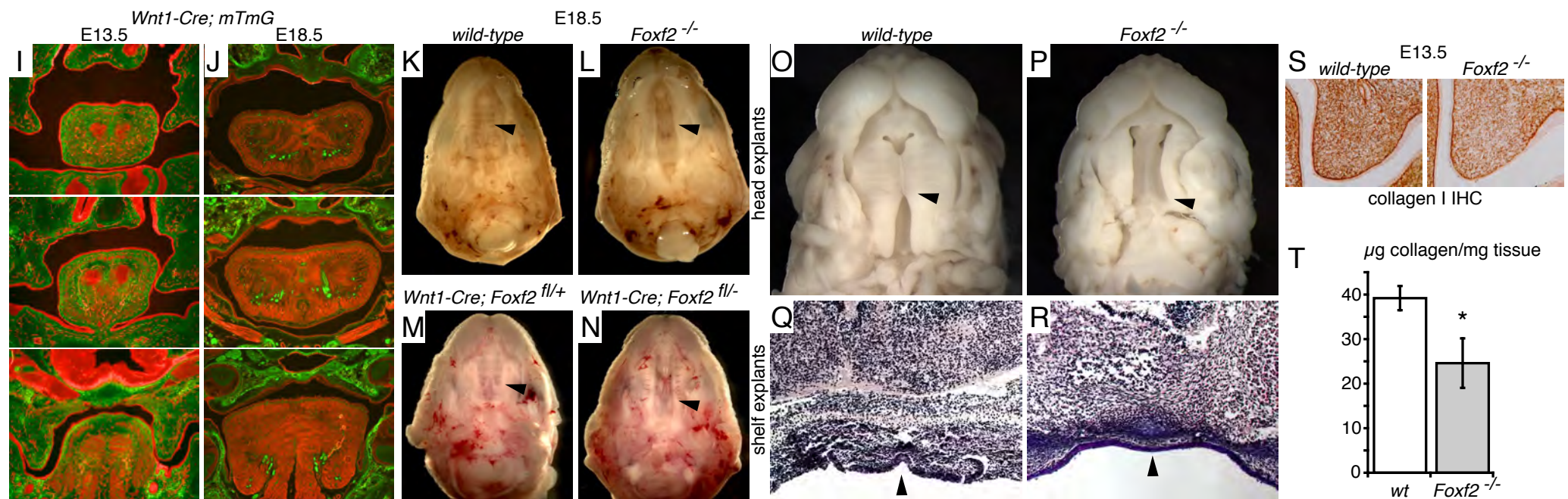
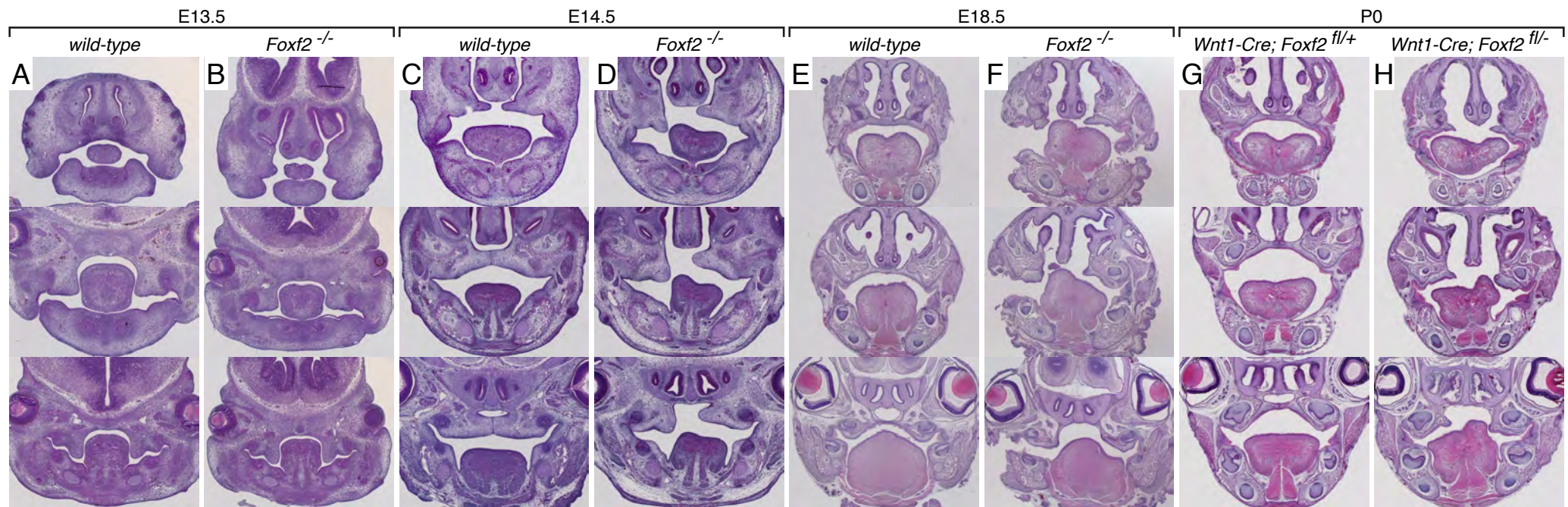
Figure 4

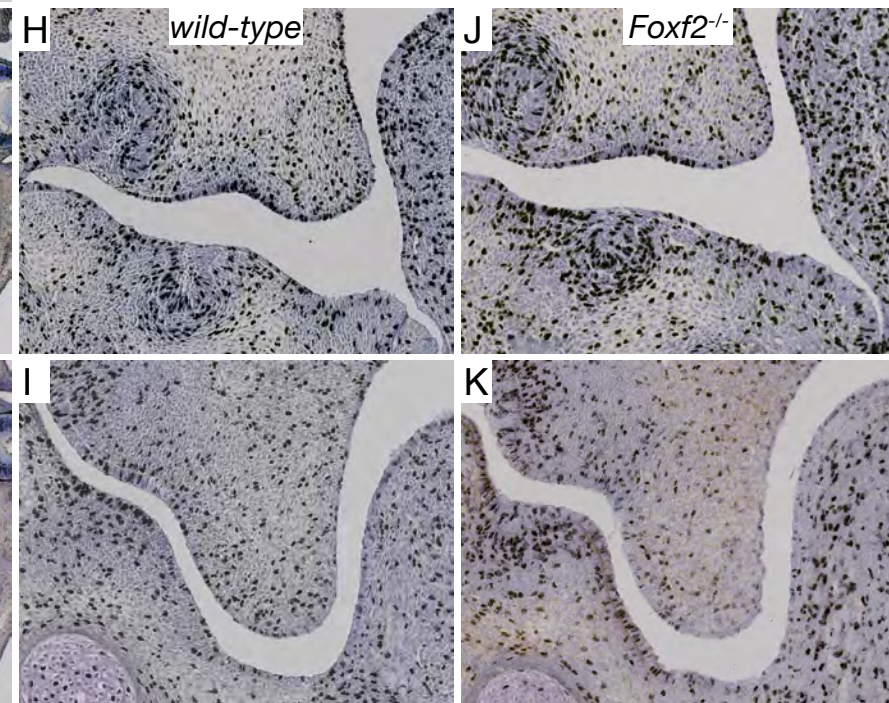
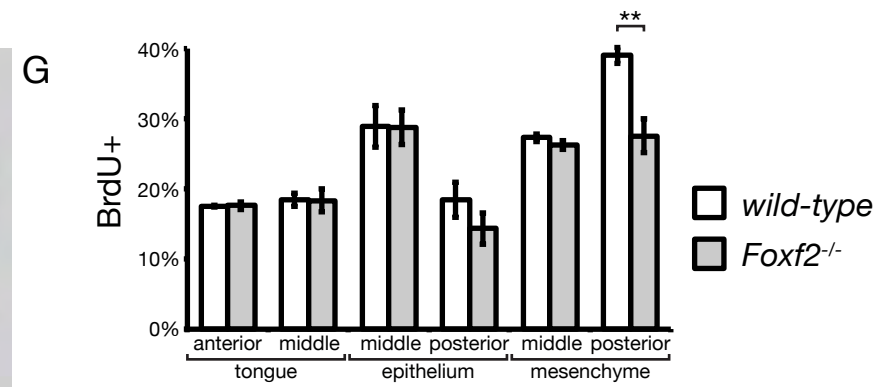
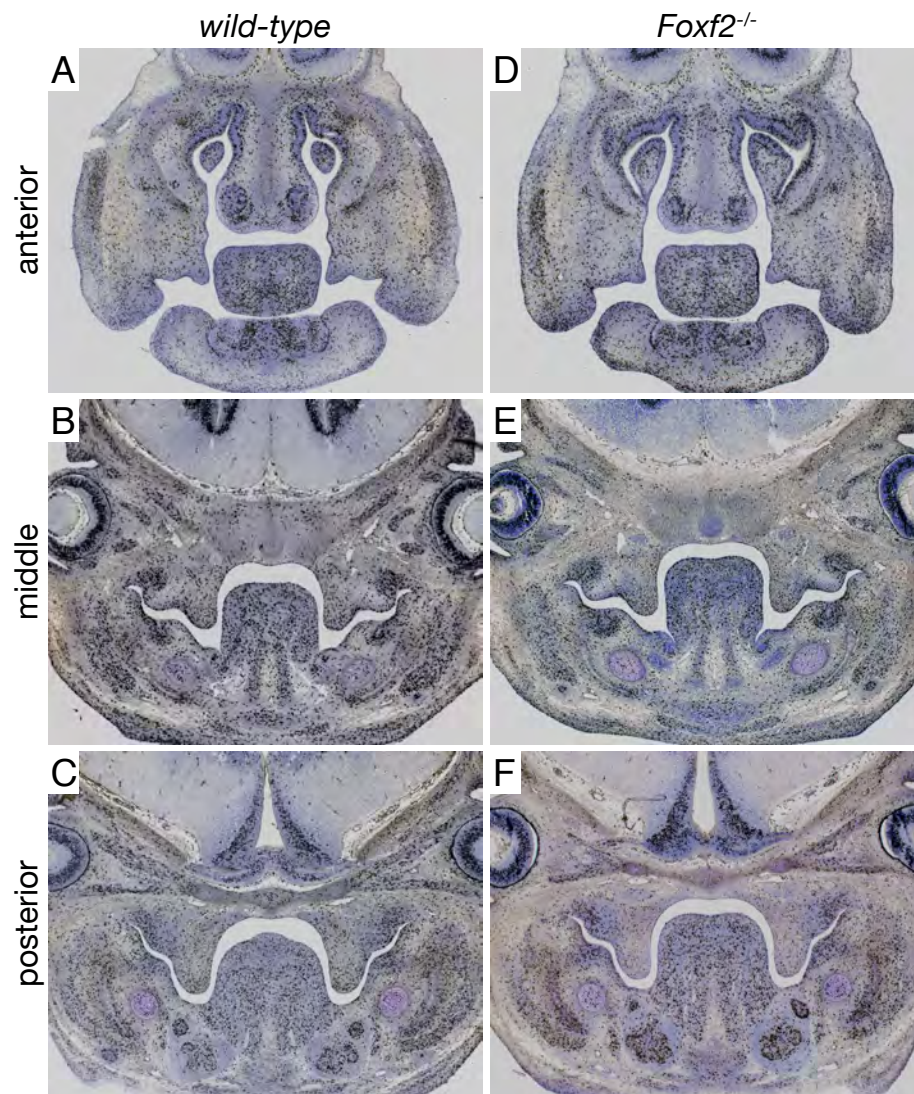
Reduced Tgf β signaling in *Foxf2*^{-/-} palatal shelves. A. Western blot with protein extracts from wild-type and *Foxf2*^{-/-} E13.5 palatal shelves showing unaltered levels of Smad2/3, p38 and β -actin (loading controls), but reduced level of Tgf β 2, decreased phosphorylation of Smad2/3, and increased phosphorylation of p38. B. qPCR quantification of mRNA in E13.5 palatal shelves showing near wild-type level in *Foxf2*^{-/-} for *Tgfb2*, but reduced concentrations for *Tgfb3*, *Itga5*, *Itgb1* and *Tnc*. C. Schematic view of fibronectin with the optional ED-A and ED-B domains (top), and two splice variants of the 46-exon *Fnl* gene. Exons 25 and 33, encoding ED-B and ED-A respectively, are indicated with asterisks. D. Western blot with protein extracts from wild type and *Foxf2*^{-/-} E13.5 palatal shelves showing moderately reduced level of fibronectin in the mutants (β -actin as loading control). E. qPCR quantification of *Fnl* mRNA showing no significant difference between genotypes of transcripts that include the ED-A exon, but a 55% reduction of Δ ED-A transcripts in *Foxf2*^{-/-} ($p=0.01$; $n=11$ wild type and 12 *Foxf2*^{-/-}).

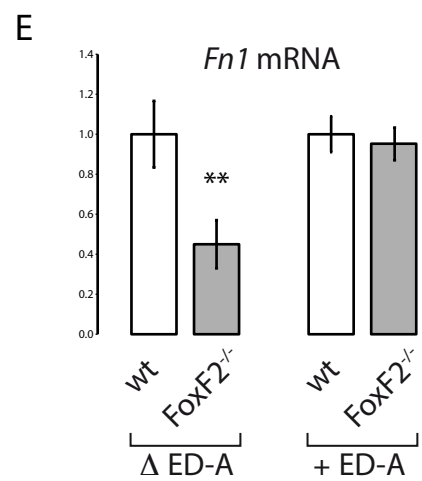
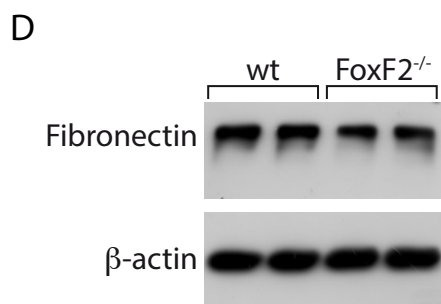
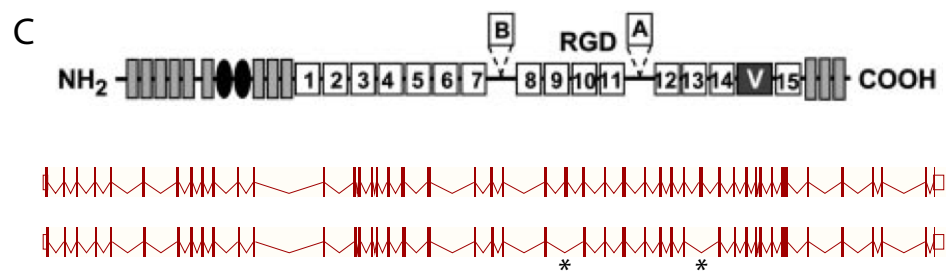
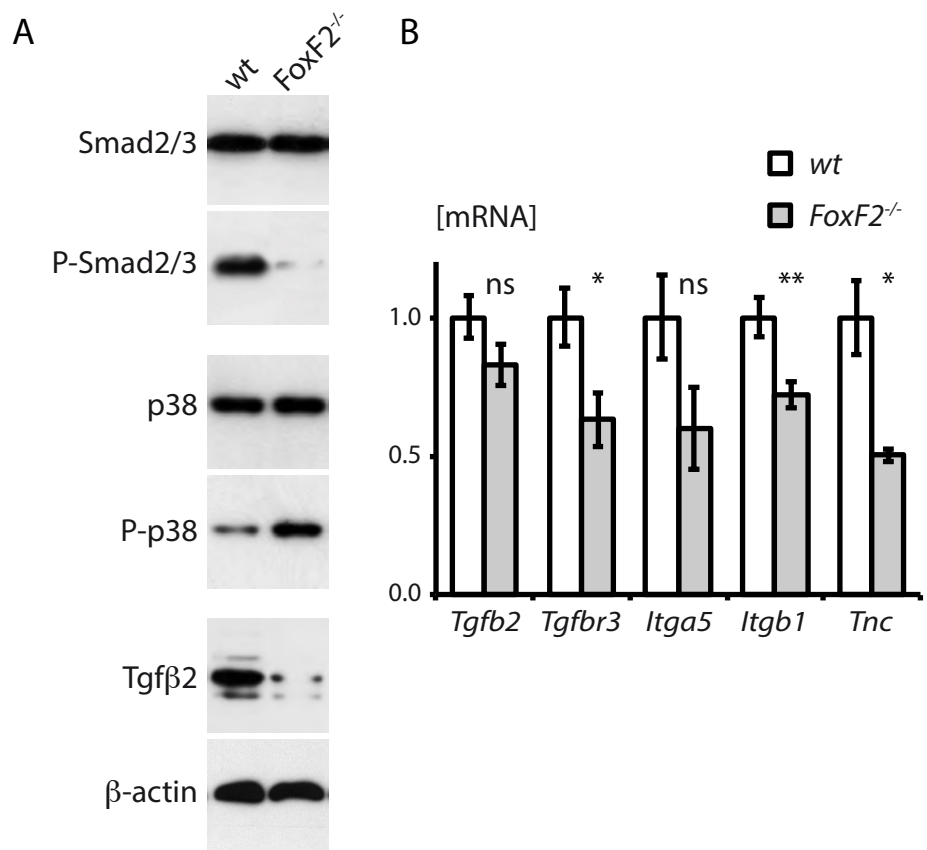
Figure 5

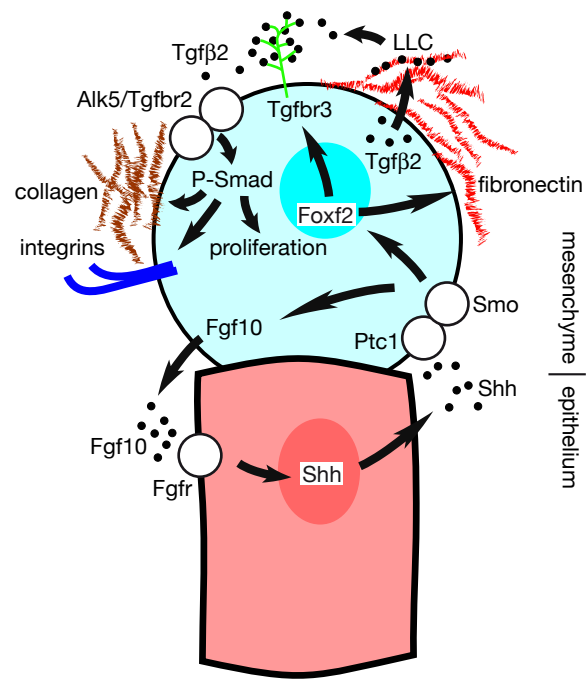
Schematic summary of the position of *Foxf2* in epithelio-mesenchymal cross talk during palatal development, and its relation to major signaling pathways essential for closure of the secondary palate. LLC = large latency complex.











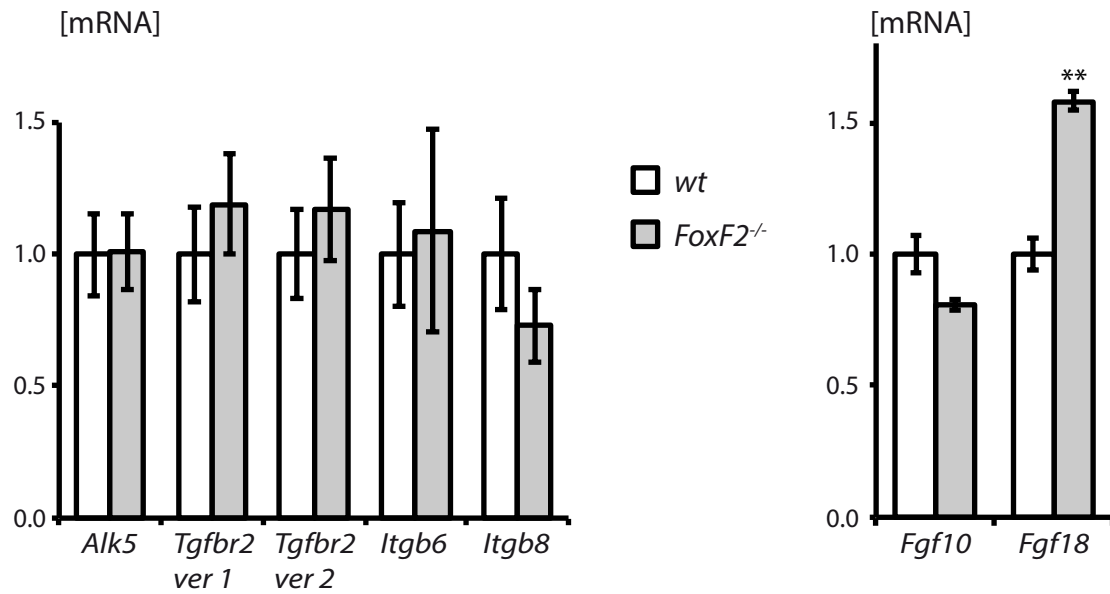


Fig S1. qPCR quantification of mRNA in E13.5 palatal shelves. Left: genes for proteins involved in Tgf-beta signaling. None of the differences between genotypes are statistically significant ($p > 0.3$). The two versions of *Tgfb2* represents two alternative splice forms. Right: genes for two Fgf:s. $p < 0.01$ for *Fgf18* and $p = 0.08$ for *Fgf10*. $n = 5$ or greater for all assays.

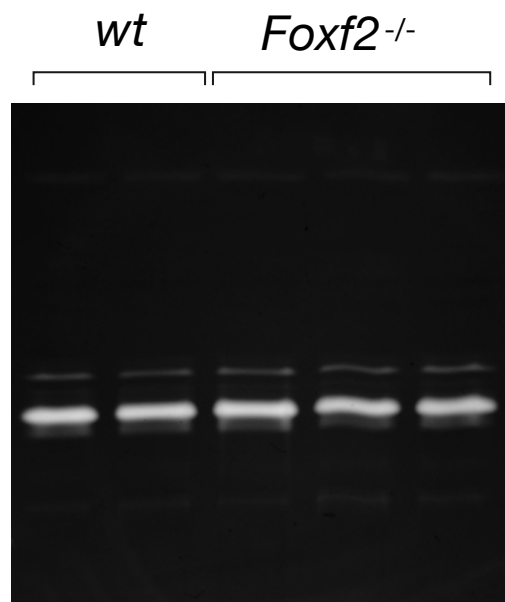


Fig S2. MMP activity assay. Protein extracts from E13.5 palatal shelves of two *wt* and three *Foxf2* knockout embryos analyzed on a 10% Zymogram gelatin gel (Novex).

Table S1

Primers for qPCR

Alk5

Forward: TCT GCA TTG CAC TTA TGC TGA

Reverse: AAA GGG CGA TCT AGT GAT GGA

Fn1 +ED-A (spans the junction of exons 33 & 34):

Forward: AAT CCA GTC CAC AGC CAT TC

Reverse: TTC ATT GGT CCT GTC TTC TC

Fn1 ΔED-A (spans the junction of exons 32 & 34):

Forward: GCA GTG ACC ACC ATT CCT G

Reverse: GGT AGC CAG TGA GCT GAA CAC

Tgfb2, primer set 1

Forward: CCC CAC ATC TCC TGC TAA TGT T

Reverse: TCT AAA GCA GTA GGC AGC ATC CA

Tgfb2, primer set 2

Forward: CTT CGA CGT GAC AGA CGC T

Reverse: GCA GGG GCA GTG TAA ACT TAT T

Tgfb2

Forward: ATG GAA GAG TGC AAC GAT TAC AT

Reverse: TGG CGC AGT TGT CAC TGA AAT

Tgfb3

Forward: CAT CTG AAC CCC ATT GCC TCC

Reverse: CCT CCG AAA CCA GGA AGA GTC

Itga5

Forward: CTT CTC CGT GGA GTT TTA CCG

Reverse: GCT GTC AAA TTG AAT GGT GGT G

Itgb1

Forward: ATG CCA AAT CTT GCG GAG AAT

Reverse: TTT GCT GCG ATT GGT GAC ATT

Itgb6

Forward: CAA CTA TCG GCC AAC TCA TTG A

Reverse: GCA GTT CTT CAT AAG CGG AGA T

Itgb8

Forward: AGT GAA CAC AAT AGA TGT GGC TC

Reverse: TTC CTG ATC CAC CTG AAA CAA AA

Fgf10

Forward: GCA GGC AAA TGT ATG TGG CAT

Reverse: ATG TTT GGA TCG TCA TGG GGA

Fgf18

Forward: CTG CGC TTG TAC CAG CTC TAT

Reverse: GAC TCC CGA AGG TAT CTG TCT

Tnc

Forward: ACG GCT ACC ACA GAA GCT G

Reverse: ATG GCT GTT GTT GCT ATG GCA